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Structural requirements for biological activity of glucagon-like peptide-I

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Glucagon-like peptide-I (GLP-I) is encoded together with glucagon by the glucagon gene and is related in its structure to the glucagon-secretin family of peptides. Three of the predicted forms of the peptide, a 37-residue long GLP-I(1–37), a 31-residue GLP-I(7–37) and a 30-residue GLP-I(7–36)amide as well as three analogs des [Gly^{37}, Arg^{36}] GLP-I(7–37), des [Gly^{37}, Arg^{36}, Gly^{35}] GLP-I(7–37) and des [His^{7}] GLP-I(7–37) were synthesized by the stepwise solid phase method. These synthetic peptides were used to define the structural domains required for the binding of GLP-I to the pancreatic beta cell. The competitive binding experiments showed that both the amino and carboxyl terminal domains of the molecule contribute to GLP-I binding. In these experiments glucagon, another peptide that stimulates insulin secretion, was a weak full agonist of GLP-I binding. Results from these studies provide further characterization of the physiological role of this new peptide.

Key words: competitive binding assays; glucagon-like peptide-I; insulin secretion; preproglucagon; solid phase peptide synthesis; Type II diabetes mellitus

Dedicated to Professor Bruce Merrifield on the occasion of his 70th birthday

The mammalian glucagon gene (1–7) encodes a 180-residue long protein that contains in addition to glucagon, located from residue 33 to 61, the sequences of two putative glucagon-like peptides, termed glucagon-like peptides I and II (GLP-I and GLP-II) (Fig. 1). The sequences of GLP-I and GLP-II were deduced to be a continuous stretch of 37 amino acids from residue 72 to 108 and 33 amino acids from residue 126 to 158, respectively in the preproglucagon sequence. They are flanked at their amino and carboxyl terminal ends by pairs of dibasic residues that are characteristic of sites that are cleaved during the posttranslational processing of prohormones. Mammalian preproglucagons are encoded by a single gene (7) and identical prohormones are expressed in pancreatic A cells and intestinal L cells (8, 9). The sequences of preproglucagons are highly conserved in all the mammalian species analyzed so far from rodent to bovine and human (1–7). Furthermore, the sequence of GLP-I is completely conserved in all of them. In fish (10), as well as in an avian species (chicken) (11), the glucagon gene encodes preproglucagon that contains the sequences of glucagon and GLP-I, but not the sequence of GLP-II. The sequences of GLP-I isolated from several different species of fish (12–16), as well as the deduced sequence of chicken GLP-I (11) show a high degree of homology to the mammalian sequence (Fig. 2). Analysis of the preproglucagon sequence suggested that mammalian GLP-I can exist in several different forms. However, we considered that posttranslational modifications would most likely take place at two sites. At the amino terminal end of the 37-residue long GLP-I(1–37) proteolytic cleavage at the arginine residue at position 6 (residue 77 in preproglucagon) will liberate a 31-residue long GLP-I. It is the sequence of this shorter GLP-I that is related to the sequences of fish and chicken GLP-I's (Fig. 2), as well as glucagon, GLP-II and other peptides with structural similarities to glucagon, such as secretin, vasoactive intestinal peptide and gastric inhibitory polypeptide

Abbreviations used: GLP, glucagon-like peptide; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; -OCH$_2$-Pam-resin, 4-(oxymethyl)phenylacetamidomethyl-copoly-(styrene-divynyl benzene) resin; Boc, t-butoxycarbonyl; Tos, tosyl; Cl-Z, p-chlorobenzyloxycarbonyl; HPLC, high pressure liquid chromatography; Chloramine T, *N*-chloro-*p*-toluensulfonamide.

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                                                                    -20
5'                                                                  Met Lys Thr Val Tyr
aaaggagctccacctgtctacacctcctctcagctcagtcccacaaggcagaataaaaaa ATG AAG ACC GTT TAC  75

                                                                   -1  1
Ile Val Ala Gly Leu Phe Val Met Leu Val Gln Gly Ser Trp Gln His Ala Pro Gln Asp
ATC GTG GCT GGA TTG TTT GTA ATG CTG GTA CAA GGC AGC TGG CAG CAT GCC CCT CAG GAC 135

                                                  15                        25
Thr Glu Glu Asn Ala Arg Ser Phe Pro Ala Ser Gln Thr Glu Pro Leu Glu Asp Pro Asp
ACG GAG GAG AAC GCC AGA TCA TTC CCA GCT TCC CAG ACA GAA CCA CTT GAA GAC CCT GAT 195

                                                  35                        45
Gln Ile Asn Glu Asp Lys Arg His Ser Gln Gly Thr Phe Thr Ser Asp Tyr Ser Lys Tyr
CAG ATA AAC GAA GAC AAA CGC CAT TCA CAG GGC ACA TTC ACC AGT GAC TAC AGC AAA TAC 255

                                                  55                        65
Leu Asp Ser Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn Thr Lys Arg Asn Arg
CTA GAC TCC CGC CGT GCT CAA GAT TTT GTG CAG TGG TTG ATG AAC ACC AAG AGG AAC CGG 315

                                                  75
Asn Asn Ile Ala Lys Arg His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser
AAC AAC ATT GCC AAA CGT CAT GAT GAA TTT GAG AGG CAT GCT GAA GGG ACC TTT ACC AGT 375

                                                  95
Asp Val Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys
GAT GTG AGT TCT TAC TTG GAG GGC CAG GCA GCA AAG GAA TTC ATT GCT TGG CTG GTG AAA 435

                                                  115
Gly Arg Gly Arg Arg Asp Phe Pro Glu Glu Val Ala Ile Ala Glu Glu Leu Gly Arg Arg
GGC CGA GGA AGG CGA GAC TTC CCG GAA GAA GTC GCC ATA GCT GAG GAA CTT GGG CGC AGA 495

                                                  135                       145
His Ala Asp Gly Ser Phe Ser Asp Glu Met Asn Thr Ile Leu Asp Asn Leu Ala Thr Arg
CAT GCT GAT GGA TCC TTC TCT GAT GAG ATG AAC ACG ATT CTC GAT AAC CTT GCC ACC AGA 555

                                                  155
Asp Phe Ile Asn Trp Leu Ile Gln Thr Lys Ile Thr Asp Lys Lys End
GAC TTC ATC AAC TGG CTG ATT CAA ACC AAG ATC ACT GAC AAG AAA TAG gaatatttcaccatt 618

cacaaccatcttcacaacatctcctgccagtcacttgggatgtacatttgagagcatatccgaagctatactgctttgc 697

atgcggacgaatacatttccctttagcgttgtgtaacccaaaggttgtaaatggaataaagttttccagggtgttgat 776

aaagtaacaactttacagtatgaaaatgctggattctcaaattgtctcctcgttttgaagttaccgccctgagattact 855

tttctgtggtataaattgtaaattatcgcagtcacgacacctggattacaacaacagaagacatggtaacctggtaacc 933

gtagtggtgaacctggaaagagaacttcttccttgaacccttttgtcataaatgcgctcagctttcaatgtatcaagaat 1012

agatttaaataaatatctcat                                                          1024

3'
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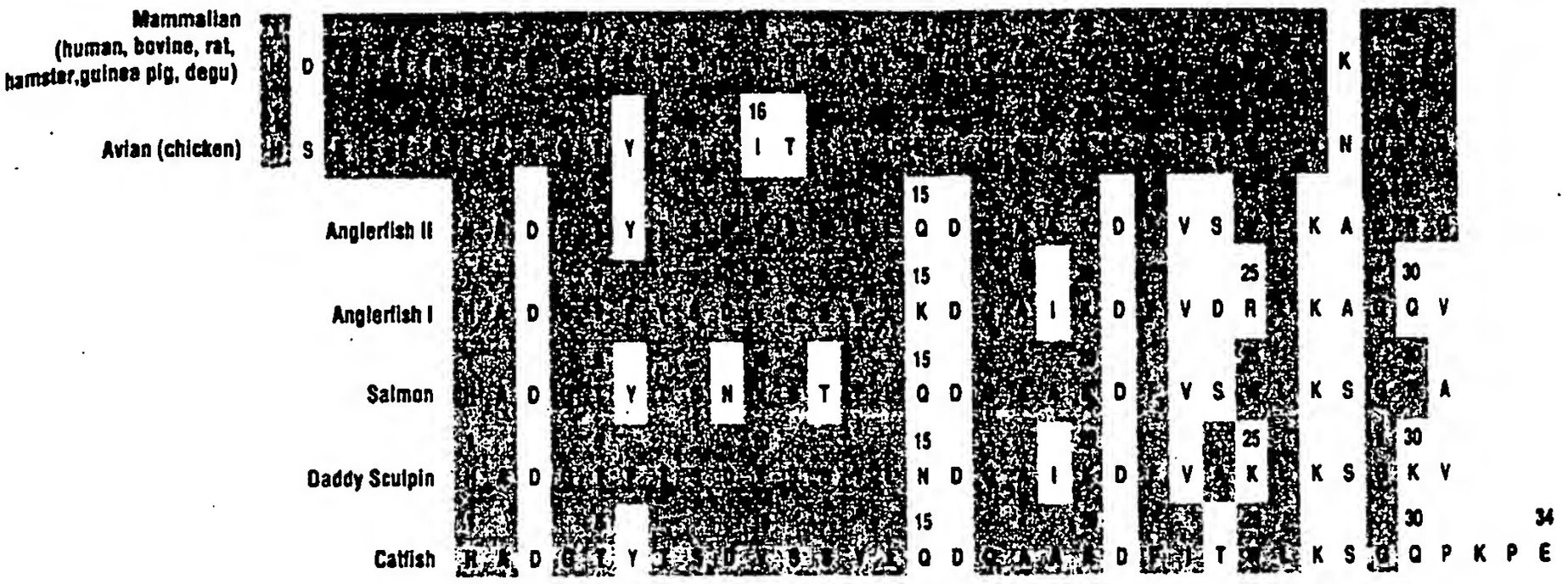


FIGURE 2

Comparison of the amino acid sequence of mammalian GLP-I with the amino acid sequences of different species of fish and avian GLP (chicken). Shaded areas represent regions of homology.

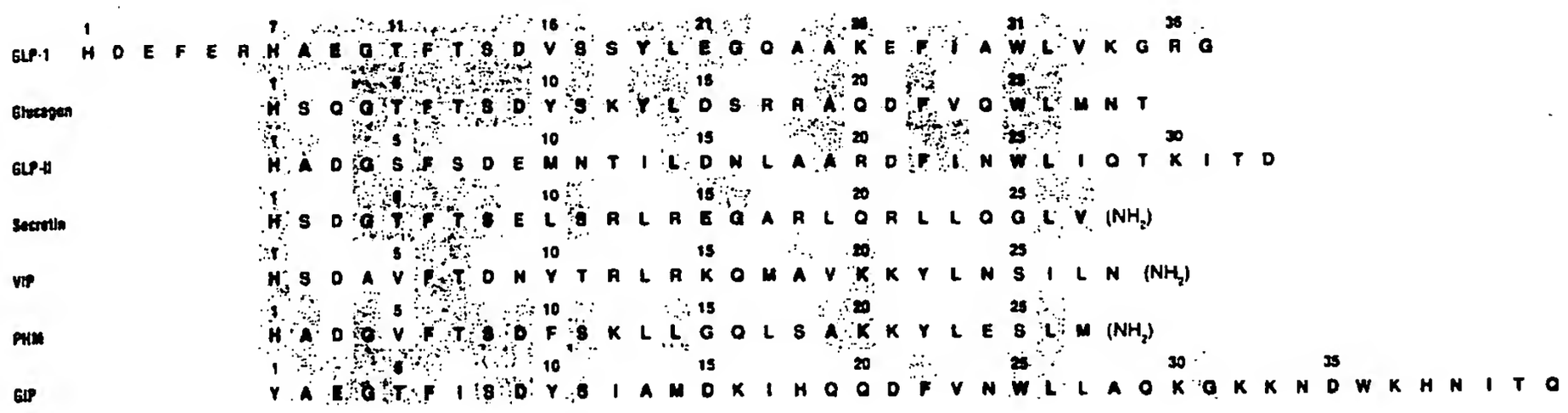


FIGURE 3

Comparison of the GLP-I sequence to the sequences of human glucagon, gastric inhibitory polypeptide and other structurally related peptides (human). Shaded areas represent regions of homology. VIP is vasoactive intestinal peptide, PHM is peptide histidine methionine amide and GIP is gastric inhibitory polypeptide.

(Fig. 3). At the carboxyl terminal end arginine residue at position 36 (residue 107 in preproglucagon) can be amidated. However, there are very few examples of basic residues that are amidated at their carboxyl terminus and we considered this modification unlikely to take place. Based on these considerations we predicted that mammalian GLP-I will be liberated from its biosynthetic precursor, preproglucagon, in at least two if not three different molecular forms: a 37-residue-long GLP-I(1–37), a 31-residue-long GLP-I(7–37) and a 30-residue-long form with an amidated carboxyl terminal arginine termed GLP-I(7–36)amide. The evolutionary conservation of the sequence of GLP-I suggested that the peptide has an essential physiological function. One of the predictions was that GLP-I might have a role in insulin secretion and it was based on the following reasoning: The major role of glucagon which is coencoded with GLP-I by the same gene is to stimulate glycolysis and gluconeogenesis through its action on the liver. This function as well as the structure of the molecule has been conserved throughout evolution from fish to man. Therefore, it seemed reasonable to assume that GLP-I, the second peptide encoded by the glucagon gene might also have a role in glucose metabolism. Furthermore, the amino acid sequence of GLP-I is related to glucagon and gastric inhibitory polypeptide (Fig. 3), two peptides that stimulate insulin secretion from beta cells in the endocrine pancreas, and the sim-

FIGURE 1

Composite nucleotide sequence of the rat preproglucagon cDNA, with the derived amino acid sequence. Pairs of dibasic residues that can be cleaved during the posttranslational processing are shown in circles with arrows adjacent to them. The sequences of GLP-I, residues 72–108 are referred to as GLP-I(1–37); residues 78–108 as GLP-I(7–37), and residues 78–107 with carboxyl terminal amidation at position 107 as GLP-I(7–36)amide.

larity in their structure suggested that GLP-I might have the same function.

This paper describes two aspects of the work whose overall aim was to define the biological role of GLP-I. The first aspect was to develop an efficient synthetic method to obtain large quantities of two of the predicted forms of the peptide, the 37-residues-long GLP-I(1–37) and the 31-residue-long GLP-I(7–37) and test our hypothesis that GLP-I stimulates insulin secretion from the pancreas. We wanted to use material obtained from a single synthesis for all the experiments performed *in vitro* model systems, animal models and human subjects. The second aspect was to generate a number of synthetic analogs of GLP-I, as well as the third predicted form of the peptide GLP-I(7–36)amide and use these compounds to define the structural domains in GLP-I sequence that are required for the expression of its biological activity.

EXPERIMENTAL PROCEDURES

Materials and methods

Assembly of the peptides was accomplished either on the Applied Biosystems Inc. peptide synthesizer model 430 or on the manual shaker (17). For the automated syntheses all the protected amino acids were purchased from Applied Biosystems Inc. Boc-protected amino acids for the large scale manual synthesis of GLP-I(7–37) and GLP-I(1–37) were purchased from Peninsula, except for Boc-Lys(Cl-Z) which was from Bachem. Boc-Gly-OCH_2-Pam-resin (0.8 mmol/g), Boc-Lys(Cl-Z)-OCH_2-Pam-resin (0.8 mmol/g) and benzhydryl amine resin (0.8 mmol/g) were from Applied Biosystems Inc. Trifluoroacetic acid, dichloromethane, diisopropylethylamine and dicyclohexylcarbodiimide were purchased also from Applied Biosystems Inc. 1-Hydroxybenztriazole was from Aldrich and was recrystallized from 70% ethanol. Analytical (0.46 x 25 cm) and semi-preparative (2.2 x 25 cm) C-18 reverse phase columns were from Vydac. Analytical ion exchange Protein-Pak DEAE 5PW column (0.75 x 7.5 cm) was from Waters. Reverse phase C-18 Delta preparative column (5.8 x 30 cm) was from Waters.

$Na^{125}I$ was from New England Nuclear, Chloramine T and sodium metabisulfite from Sigma.

Tissue culture media and antibiotics were from Gibco.

Peptide hydrolyses were performed in 6 N HCl containing 2-mercaptoethanol for 22 h at 110°. Amino acid analyses were performed on the Beckman model 6300 amino acid analyzer. High pressure liquid chromatography was from Waters. Sequencing of the synthetic peptides was performed on the Beckman 890M Sequencer. Amino acid derivatives were analyzed on reverse phase ultrasphere ODS column (0.46 x 15 cm) (Beckman) HPLC system (Beckman) with a gradient of 0.2 M lithium acetate (buffer A) and 85% methanol in buffer A (25–100%) formed in 20 min at flow rate 1.75 mL/min. Mass spectra were determined by the electrospray method at the Mass spectra facility at The Rockefeller University.

For the clinical studies pure synthetic GLP-I(7–37) was aliquoted (100 μg) together with mannitol (500 μg) under sterile conditions and was stored in dark at 4°.

Synthetic glucagon that was used in the competitive binding experiments was synthesized on the Applied Biosystems Inc. instrument using synthetic strategy and purification scheme as previously described (18).

Solid phase peptide synthesis of different forms of GLP-I's and their analogs

General. The synthetic strategy was based on the use of 4-(oxymethyl) Pam-resin support and N-α-Boc and benzyl based side chain protection. The indole ring of tryptophan was protected with the formyl group and the imidazole ring of histidines with the tosyl group. One synthetic cycle consisted of the following steps: (1) CH_2Cl_2 wash and pre-swell (3 x 1 min); (2) deprotection with 50% F_3CCOOH/CH_2Cl_2 (1 x 1 min prewash, 1 x 20 min); (3) CH_2Cl_2 wash (5 x 1 min); (4) neutralization with 5% iPr_2EtN/CH_2Cl_2 (3 x 1 min); (5) CH_2Cl_2 wash (5 x 1 min); (6) Boc-amino acid coupling using different protocols and reaction times as described below for the individual peptides; (7) CH_2Cl_2 wash (5 x 1 min). Additional steps that were performed in the syntheses of different peptides are described below. The cleavage of the peptides from the resin and deprotection of all the protecting groups was accomplished by the two step low/high HF (19). Prior to the HF treatment amino terminal Boc-group was deprotected with 50% F_3CCOOH/CH_2Cl_2 (1 x 1 min prewash, 1 x 20 min). Following the HF cleavage peptides were dissolved in 50% CH_3COOH/H_2O (20 mL per 1 g protected peptide resin) and the aqueous phase was extracted with ether (3 x 20 mL). Aqueous phase was diluted with water to a final concentration of 10% CH_3COOH and material was recovered by lyophilization.

GLP-I (7–37). For the assembly of the peptide chain manual shaker was used as originally described (17). Synthesis started with 3.6 g of Boc-Gly-OCH_2-Pam-resin (substitution 0.8 mmol/g). At the beginning of the synthesis all the reagents were added in 40 mL. This volume increased with the length of the peptide chain and was 100 mL towards the end of peptide assembly. For each coupling 2.5-fold excesses of protected amino acid and DCC were used, except for glutamine residues that were coupled with 2.5-fold excess of DCC and 1-hydroxybenzotriazole. Coupling times were 90 min. Symmetrical anhydrides were used when the couplings were to a glutamine residue to prevent cyclization of its N-α amino group (20), or if more than two couplings were required for quantitative incorporation of the protected amino acid. Each coupling step was monitored by the quantitative ninhydrin reaction (21). Samples of

the protected peptide resin were removed after the first coupling and the coupling yield was determined while the second coupling was still in progress. If the coupling yield was more than 99.5% the synthesis proceeded to the next residue. However, if it was less than 99.5% the synthesis would not proceed until the result from the ninhydrin test following the second coupling was obtained. Depending on the extent of incorporation either a third coupling would be performed or the synthesis would continue to the next residue. Upon completion of the synthesis 14.1 g of protected peptide resin were obtained, representing 95% of the theoretical weight gain. This amount of 14.1 g includes 0.5 g of protected peptide resin removed for ninhydrin monitoring.

GLP-I(1–37). The assembly was accomplished starting with 2 g of protected GLP-I(7–37) resin using the same coupling protocol as described for the synthesis of GLP-I(7–37).

GLP-I(7–36)amide. Synthesis was performed on benzhydrylamine resin (0.5 g, substitution 0.8 mmol/g). For the assembly of the peptide chain Applied Biosystems instrument was used. Results from the ninhydrin monitoring of the manual solid phase synthesis of GLP-I(7–37) (Table 2) were used as a guide for the number of couplings needed for incorporation of each protected amino acid into the peptide chain. Protected amino acids were coupled for 60 min as symmetrical anhydrides without monitoring the extent of coupling reactions. Exception was an amino terminal histidine residue for which ninhydrin test showed that four couplings were needed for quantitative incorporation in agreement with the results obtained from the manual synthesis of GLP-I(7–37) (Table 2).

des [Gly^{37}, Arg^{36}] GLP-I(7–37), des [Gly^{37}, Arg^{36}, Gly^{35}] GLP-I (7–37) and des [His^{7}] GLP-I (7–37). Syntheses were performed on Boc-Gly-OCH$_2$-Pam resin (0.5 g, substitution 0.8 mmol/g) for des [Gly^{37}, Arg^{36}] GLP-I(7–37) and des [His^{7}] GLP-I(7–37) and Boc-Lys(Cl-Z)-OCH$_2$-Pam-resin (0.5 g, substitution 0.8 mmol/g) for des [Gly^{37}, Arg^{36}, Gly^{35}]. The assembly of the peptides was accomplished on the Applied Biosystems instrument in the same manner as described for the synthesis of GLP-I(7–36)amide.

Purification of the different forms of synthetic GLP-I's and their analogs

General. Synthetic peptides were purified on the semipreparative C-18 reverse phase HPLC or Delta-preparative C-18 reverse phase column in a Waters HPLC system depending on the amount of peptide that needed to be purified. Prior to the application on the Delta-preparative column peptides were desalted on Sephadex G-10 (1 × 50 cm) in 10% acetic acid. They were eluted from the C-18 reverse phase columns with a gradient of 0.1% TFA/water and 0.1% TFA/acetonitrile (35–65%) formed in 30 min at a flow rate 2 mL/min in the semipreparative column and 50 mL/min in the Delta-preparative column. Appropriate fractions were pulled together, neutralized with 100 mM ammonium hydroxide and material was recovered by lyophilization.

GLP-I(7–37). For the studies involving human subjects pure GLP-I(7–37) (140 mg) was applied on a second Delta preparative column and eluted with a solvent gradient of triethylammonium phosphate pH 7 in water (buffer A) and 60% buffer A in acetonitrile (buffer B) (45–68%) formed in 45 min with a flow rate of 50 mL/min. The peak four fractions were pulled together and lyophilized giving 20 mg of peptide (14.3% of the total applied).

Analytical characterization of synthetic GLP-I's and their analogs

The homogeneity of the synthetic GLP-I peptides was checked using two different chromatographic modes of separation. The first one was reverse phase C-18 and the second one was ion exchange Protein-Pak DEAE 5PW HPLC. Exception was des [His^{7}] GLP-I(7–37) which was applied on the analytical reverse phase C-18 HPLC and eluted with two different solvent systems. Results are listed in Table 1. As seen from Table 1 in both chromatographic systems we were able to define solvent and gradient conditions in which the three predicted forms of GLP-I's and the carboxyl terminal analogs can be separated from each other.

Acid hydrolysis in 6 N HCl containing 2-mercaptoethanol followed by amino acid analysis gave the following ratio of amino acids:

GLP-I(7–37): Asp 1.0(1), Thr 1.9(2), Ser 2.7(3), Glu 4.0(4), Gly 4.0(4), Ala 4.0(4), Val 2.1(2), Ile 1.0(1), Leu 2.1(2), Tyr 1.0(1), Phe 2.0(2), His 1.1(1), Lys 2.0(2), Arg 1.0(1);

GLP-I(1–37): Asp 1.9(2), Thr 2.0(2), Ser 2.5(3), Glu 5.9(6), Gly 4.3(4), Ala 4.1(4), Val 2.3(2), Ile 1.1(1), Leu 2.1(2), Tyr 0.9(1), Phe 3.1(3), His 1.8(2), Lys 2.1(2), Arg 2.2(2);

GLP-I(7–36)amide: Asp 1.0(1), Thr 1.9(2), Ser 2.8(3), Glu 4.2(4), Gly 3.1(3), Ala 4.1(4), Val 2.0(2), Ile 1.0(1), Leu 2.1(2), Tyr 1.0(1), Phe 2.0(2), His 1.0(1), Lys 2.0(2), Arg 1.1(1);

des [Gly^{37}, Arg^{36}] GLP-I (7–37): Asp 1.1(1), Thr 1.8(2), Ser 2.7(3), Glu 4.0(4), Gly 3.2(3), Ala 4.0(4), Val 2.0(2), Ile 1.0(1), Leu 2.1(2), Tyr 1.0(1), Phe 2.1(2), His 1.0(1), Lys 2.1(2);

des [Gly^{37}, Arg^{36}, Gly^{35}] GLP-I (7–37): Asp 1.0(1), Thr 1.7(2), Ser 2.6(3), Glu 4.1(4), Gly 2.1(2), Ala 4.1(4), Val 2.1(2), Ile 1.0(1), Leu 2.1(2), Tyr 1.0(1), Phe 2.1(2), His 1.0(1), Lys 2.1(2);

des [His^{7}] GLP-I (7–37): Asp 1.0(1), Thr 1.8(2), Ser 2.7(3), Glu 4.1(4), Gly 4.1(4), Ala 4.0(4), Val 2.0(2), Ile 1.0(1), Leu 2.1(2), Tyr 1.0(1), Phe 2.1(2), Lys 2.1(2),

TABLE I

Analytical HPLC properties of synthetic GLP's and their analogs

Peptide	HPLC retention times (min)		
	1[a]	2[b]	3[c]
GLP-I (7–37)	11.5	11.1	
GLP-I (1–37)	8.9	16.5	
GLP-I (7–36)amide	13.5	8.2	
des [Gly[37], Arg[36]]GLP-I (7–37)	10.2	13.6	
des [Gly[37], Arg[36], Gly[35]]GLP-I (7–37)	7.6	13.9	
des [His[7]]GLP-I (7–37)	11.5	–	18.9

[a] Analytical reverse phase C-18 column. Buffer A: Triethylammonium phosphate, pH 7; Buffer B: 40% buffer A in 60% acetonitrile; Elution was with a gradient 45–68% buffer B formed in 30 min at a flow rate 1 mL/min. During the 2-yr period of chromatographic analyses in this solvent systems we observed 1–2 min variation in the elution positions of these peptides depending on the lot of the reverse phase C-18 column. However, the relative differences in elution positions between the peptides remained the same.

[b] Analytical ion exchange Protein-Pak DEAE 5PW column. Buffer A: 20 mM Tris HCl pH 8.5; Buffer B: 20 mM Tris HCl pH 8.5 containing 0.5 M NaCl. Elution was with a gradient 0–70% buffer B formed in 25 min at flow rate 1 mL/min.

[c] Analytical reverse phase C-18 column. Buffer A: 0.1% TFA/water; Buffer B: 0.1% TFA/acetonitrile. Elution was with a gradient 20–70% buffer B formed in 30 min at flow rate 1 mL/min.

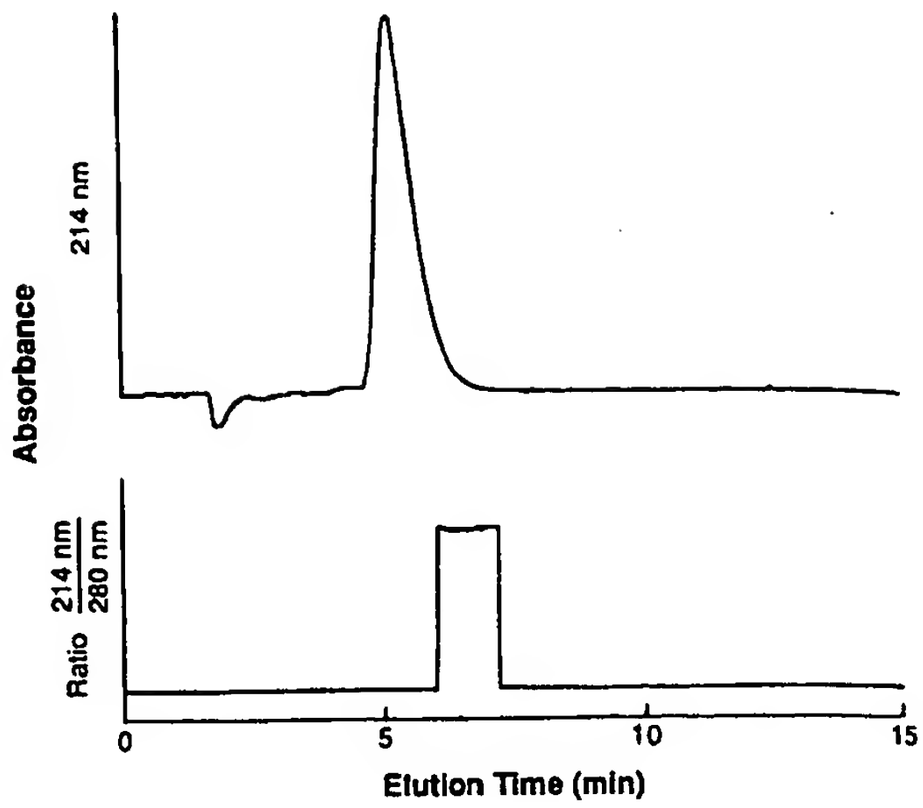


FIGURE 4

UV ratio plot at 214 nm/280 nm of pure synthetic GLP-I(7–37) after the second application on Delta preparative C-18 (5.7 cm x 30 cm) on the Waters HPLC. GLP-I(7–37) (10 μg) was applied on the analytical reverse phase C-18 HPLC and eluted under isocratic conditions of 27% organic phase. (0.1% TFA in water was aqueous phase and 0.1% TFA in acetonitrile was the organic phase). An identical plot was obtained at 229/280 nm absorbance ratio.

Arg 0.9(1). The values for threonine and serine are not corrected.

UV scan of all six peptides in the range 220–360 nm had a maximum absorption at 282 nm. The homogeneity of GLP-I(7–37) was additionally confirmed by UV ratio plots at 214/280 nm and 229/280 nm (Fig. 4).

Sequencing of the peptides gave the following results. GLP-I (1–37): All 37 residues were present in the expected sequence. Preview was observed at residues 2 (3% of Glu from residue 3), 3 (8% of Phe from residue 4), 6 (5% of His from position 7), 11 (8% of Phe from residue 12), 15 (8% of Val from residue 16), 18 (4% of Tyr from residue 19), 19 (4% of Leu from position 20), 27 (12% of Phe from position 28) and 32 (4% of Val from position 33).

GLP-I(7–37): All the amino acids were present in the expected sequence up to position 35 (29 residues into the sequence) when there was a considerable drop in the recovery yield. However, the two missing carboxyl terminal amino acids, arginine and glycine were clearly detectable in the sequence of GLP-I(1–37) which was obtained from the same synthesis as GLP-I(7–37), as described above. Preview was observed at residues 8 (2% of Glu from residue 9), 9 (2% Gly from residue 10), 11 (1% of Phe from residue 12), 15 (1% of Val from residue 16), 16 (5% of Ser from residue 17), 17 (1% of Tyr from residue 19), 18 (2% of Tyr from residue 19), 19 (6% of Leu from residue 20, 3% of Glu from residue 21), 20 (7% of Glu from residue 21), 22 (2% of Gln from residue 23), 24 (3% of Ala from residue 25), and 27 (3% of Phe from residue 28).

des [Gly[37], Arg[36]] GLP-I (7–37): All residues were present in the expected sequence. Preview was observed at residues 8 (3% of Glu from residue 9), 11 (7% of Phe from residue 12), 14 (7% of Asp from residue 15), 15 (7% of Val from residue 16), 18 (6% of Tyr from residue 19), 19 (5% of Leu from residue 20), 20 (9% of Glu from residue 21), 21 (4% of Gly from residue 22), 22 (7% of Gln from residue 23, 2% of Ala from residue 24), 23 (7% of Ala from residue 24), 25 (5% of Lys from residue 26), 26 (3% of Glu from residue 27), 27 (6% of Phe from residue 28), and 29 (9% of Ala from residue 30).

des [His[7]] GLP-I (7–37): All residues were present in the expected sequence. Preview was observed at residues 8 (less than 1% of Glu from residue 9), 9 (2% of Gly from residue 10), 11 (1% of Phe from residue 12), 14 (2% of Asp from residue 15), 15 (2% of Val from residue 16), 18 (3% of Tyr from residue 19), 19 (3% of Leu from position 20), 20 (3% Glu from residue 21), 21 (4% of Gly from residue 22), 22 (2% of Gln from residue 23), 23 (2% of Ala from residue 24), 27 (3% of Phe from residue 28), and 32 (1% of Val from residue 33).

The amount of preview observed in all of the above sequencing runs was at the level found in the sequencing of myoglobin that was used as a control.

Molecular weights determined by the mass spectra electrospray method were as follows: GLP-I(7–37): 3354.5 (Calculated 3355.7); GLP-I(1–37): 4169.7 (Calculated 4169.3); GLP-I(7–36)amide: 3298.8 (Calculated 3297.7); des [Gly^{37}, Arg^{36}] GLP-I(7–37): 3141.7 (Calculated 3142.1); des [Gly^{37}, Arg^{36}, Gly^{35}] GLP-I(7–37): 3085.1 (Calculated 3085.4); des [His^{7}] GLP-I(7–37): 3217.9 (Calculated 3218.3).

Iodination of GLP-I (7–37)

GLP-I(7–37) was iodinated on the single tyrosine residue by the Chloramine T method as described (22). Briefly, synthetic GLP-I(7–37) (4 μg) in 0.5 M phosphate buffer pH 7.5 (100 μL), Na^{125}I (1 mCi) and Chloramine T (10 μL from stock solution 2.5 mg per mL) were mixed together for 15 s at room temperature. Reaction was terminated by the addition of sodium metabisulfite (25 μL from stock 2.5 mg per mL). 0.1% BSA in 0.1% TFA/water (1 mL) was added as carrier and reaction mixture was applied on the Sep-Pak C-18 cartridge (Waters). The cartridge was washed first with 0.1% TFA/water (20 mL) and then with 60% acetonitrile containing 0.1% TFA (3 mL) to elute the radioiodinated peptide mixture. Material was recovered by lyophilization. Iodination yield calculated from the amount of radioactivity added varied and was in the order of 25–50%. Iodination products were purified on reverse phase C-18 column (0.4 x 30 cm) (Vydac) with a gradient of 0.1% TFA in water and 0.1% TFA in acetonitrile (35–55%) formed in 30 min, with a flow rate 2 mL/min. Outlet of the column was connected to a fraction collector and 0.2 min fractions were collected. Chromatographic separation was followed by measuring the radioactivity in the eluted fractions on the gamma counter. Under these conditions monoiodinated Tyr^{19} GLP-I(7–37) eluted at 15.2 min and diiodinated form at 16.8 min. The assignment of the peaks was confirmed by their digestion with pronase (10 mg/mL) in 20 mM Tris-HCl buffer pH 7.5 (36 h, 32°). Mono- and di-iodinated tyrosine residues were separated on the reverse phase C-18 column (Vydac) with a gradient of 0.1% TFA in water and 0.1% TFA in acetonitrile (0–50% B) formed in 30 min at flow rate 1 mL/min. Monoiodinated tyrosine elutes at 12.6 min and diiodinated at 16.0 min. Neither of the peaks contained mono- or di-iodinated histidines which would have eluted at 2.4 min and 8.7 min respectively. Material was stored at −20°.

Binding assay

RIN 1046-38 cells were a generous gift from Dr. W.L. Chick. They were grown in Dulbecco's modified Eagle's medium (DMEM), containing 4.5 g glucose per liter, supplemented with 10% fetal bovine serum, 100 μg streptomycin per mL and 100 units of penicillin per mL under an atmosphere of 5% CO2. For the binding assay cells were plated in 24-well plates (Falcon) and the assay was performed 24–48 h later when they were about 50% confluent. Assay buffer was 50 mM Tris base pH 7.5 containing 10 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, 20 mM Hepes, 1% BSA, 11 mM glucose. GLP-I peptides in the concentration range 10^{-6} M–10^{-12} M and ^{125}I-Tyr^{19} GLP-I(7–37) (100000 cpm, 21 fmol) were added to the cells in a final volume of 0.5 mL and were incubated for 16–18 h at 4°. Each concentration was added in triplicate wells and for most of the peptides duplicate plates were used. At the end of the incubation period supernatant was discarded and cells were lysed with 1 N NaOH (200 μL). Wells were washed two more times with 1 N NaOH (200 μL) and the washes combined. Cell-associated radioactivity was measured on the gamma counter. Variation between triplicate wells was 2–5%. Total binding was determined as binding of monoiodinated ^{125}I-Tyr^{19} GLP-I(7–37) in the absence of any peptide added and nonspecific binding as the amount bound in the presence of 10^{-6} M peptide. Specific binding was calculated as the difference between the total and nonspecific binding and was expressed as the percent of the total radioactivity added. Stock solutions of synthetic peptides used in the assay to displace the binding of ^{125}I-Tyr^{19} GLP-I(7–37) were at concentrations of 10^{-5} M and serial dilutions were made from it. The concentrations of the stock solutions were determined by acid hydrolysis followed by amino acid analysis.

RESULTS AND DISCUSSION

Solid phase peptide syntheses and purifications

Although there is a large number of analytical data on coupling yields of Boc-protected amino acids there are no general rules that will predict the extent of their incorporation into the peptide chain. To ensure quantitative incorporation (> 99.5%) of each residue into the peptide chain during the large scale solid phase syntheses of GLP-I(7–37) and GLP-I(1–37) we determined each coupling yield using the quantitative ninhydrin test (21). Results from the ninhydrin determinations for GLP-I(7–37) synthesis are shown in Table 2. As seen from Table 2 starting with the tryptophan residue, which is the sixth residue that was incorporated into the peptide chain, there was a stretch of about 10 residues that required a third coupling for quantitative incorporation. This was followed by a stretch of about 12 residues that were incorporated in a single coupling. Quantitative incorporation of Boc-His(Tos) 31-residues into the peptide chain required four couplings.

Protected GLP-I(7–37) peptide-resin (2 g) was used as a starting material for the synthesis of GLP-I(1–37). Quantitative ninhydrin determinations showed that single couplings were sufficient for quantitative incorporation of all the residues including the amino terminal Boc-His(Tos).

We did not monitor the coupling yields during the syntheses of GLP-I(7–36)amide and the three GLP-

TABLE 2

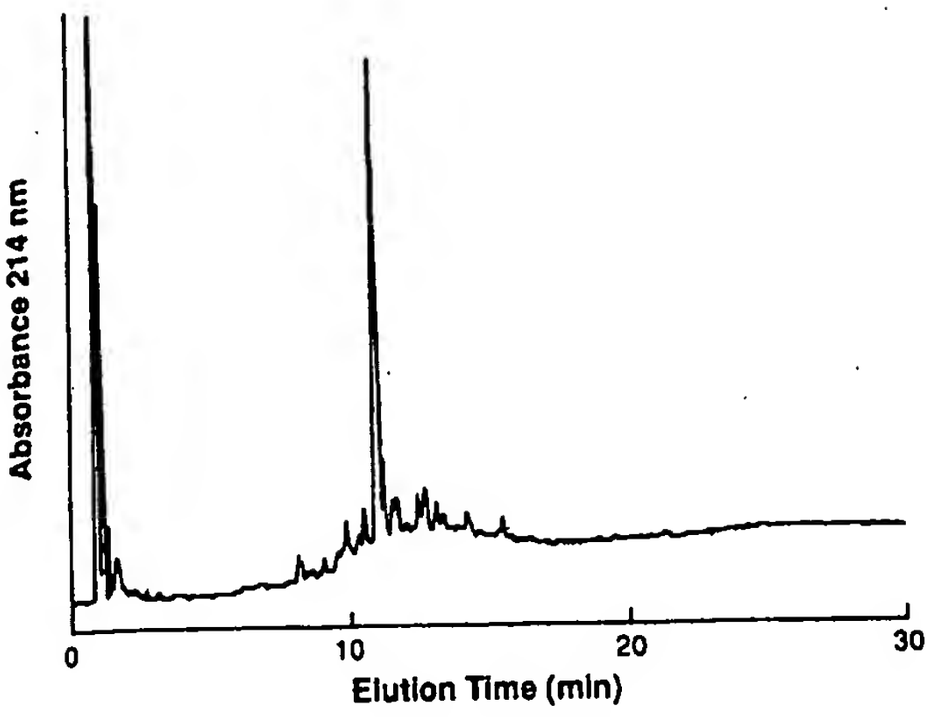
Coupling yields obtained by quantitative ninhydrin test (21) during solid phase peptide synthesis of GLP-I (7–37)

Residue number	Amino acid	Method of coupling	% of free amino group left	Residue number	Amino acid	Method of coupling	% of free amino group left
30	Arg	DCC	0.4	17	Gln	DCC/HOBt	3.0
		DCC	0.2			DCC/HOBt	0.3
						DCC/HOBt	0.2
29	Gly	DCC	0.2				
		DCC	0.1	16	Gly	symm	0.6
						anhyd	0.1
28	Lys	DCC	0.1				
				15	Glu	DCC	0.3
27	Val	DCC	0.5			DCC	0.1
		DCC	0.2				
				14	Leu	DCC	0.3
26	Leu	DCC	0.4			DCC	0.1
		DCC	0.3				
				13	Tyr	DCC	0.2
25	Trp	DCC	1.7	12	Ser	DCC	0.2
		DCC	0.5	11	Ser	DCC	0.1
		DCC	0.6				
				10	Val	DCC	0.3
24	Ala	DCC	0.5			DCC	0.1
		DCC	0.3				
				9	Asp	DCC	0.1
23	Ile	DCC	7.1	8	Ser	DCC	0.1
		DCC	1.2				
		symm anhyd	0.5	7	Thr	DCC	0.4
						DCC	0.1
22	Phe	symm	1.2				
		anhyd	0.3	6	Phe	DCC	0.2
				5	Thr	DCC	0.2
21	Glu	DCC	5.3	4	Gly	DCC	0.1
		symm	0.7	3	Glu	DCC	0.1
		anhyd		2	Ala	DCC	0.1
20	Lys	DCC	1.8				
		DCC	0.5	1	His	DCC	8.0
		DCC	0.1			DCC	6.3
						symm	1.0
19	Ala	DCC	0.4			anhyd	0.4
		DCC	0.1				
18	Ala	DCC	2.9				
		DCC	0.3				

I(7–37) analogs on the Applied Biosystems instrument, because the amount of peptide-resin that we needed to use for the ninhydrin determinations would have considerably reduced the final yield of the peptides. This was also not necessary because we had already determined the number of couplings that were required for quantitative incorporation of each residue for the parent compound GLP-I(7–37).

Chromatographic separation of the crude GLP-I(7–37) on the reverse phase C-18 HPLC in two different solvent systems is shown in Fig. 5A and B. In both solvent gradients the main peak accounted for 70–80% of the total material. The main peak had the correct amino acid composition and sequence. Crude GLP-I(1–37), GLP-I(7–36)amide as well as the carboxyl and amino terminal shortened analogs had similar distribution of the main peak and the two byproducts as shown in Fig. 5A and B for GLP-I(7–37).

In a typical purification of GLP-I(7–37), from 2 g of protected peptide resin 148 mg of pure peptide was obtained representing 6% of the theoretical yield. This amount of peptide was sufficient for all the needed characterizations and biological applications. (Calculation of the theoretical yield of 2.63 g was based on a 70% cleavage yield from low/high HF and 70% of the desired synthetic peptide being in the main peak). The



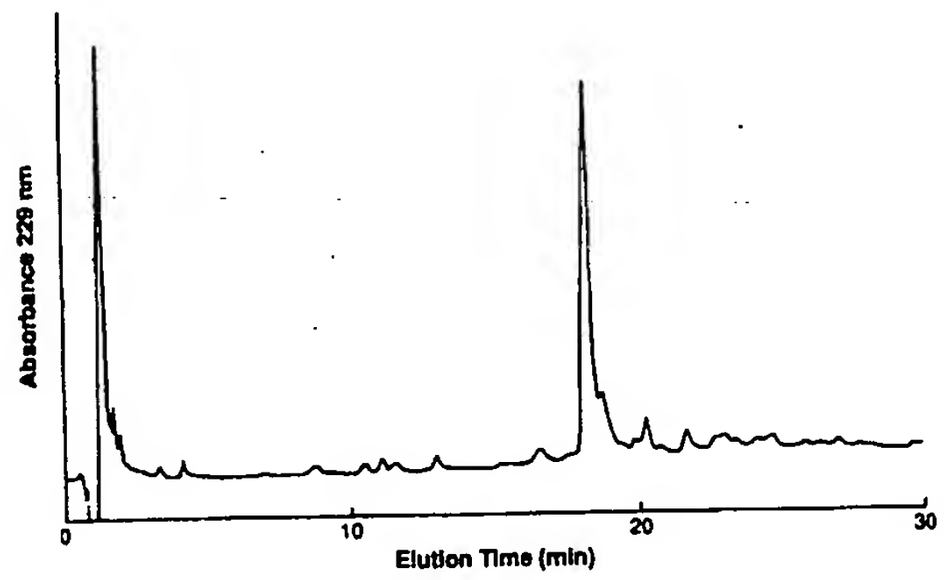


FIGURE 5

Analytical reverse phase C-18 HPLC (Vydac, 0.4 × 30 cm) of the crude GLP-I(7–37) directly after cleavage and deprotection in low/high HF. Elution was with a gradient of 0.1% TFA/water and 0.1% TFA/acetonitrile (20–70%) formed in 30 min at a flow rate of 1 mL/min (Panel A) or with a gradient of triethylammonium phosphate pH 5.2 in water (buffer A) and 40% buffer A in 60% acetonitrile (45–68%) formed in 30 min at flow rate 1 mL/min (Panel B). The peak eluting at the void volume in both chromatograms is the solvent peak and it did not contain any peptide as determined by acid hydrolysis and amino acid analysis.

entire amount of synthetic material recovered from a Delta preparative column was 244 mg (11%). The disconcerting loss of so much material may be partially due to the nature of the peptide and not entirely due to the nature of the column packing and its size.

Stability of synthetic GLP-I (7–37)

Because synthetic GLP-I(7–37) was intended also for clinical studies with human subjects its stability under different conditions was studied. Incubation of GLP-I(7–37) at pH 7 at 55° up to 72 h did not give rise to any degradation products as detected by analytical reverse phase C-18 HPLC chromatography in the two solvent systems shown in Fig. 5A and B. Only after 144 h were small amounts of degradation products detected. Incubation at either pH 1 or pH 10 for 24 h gave rise to small amounts of byproducts eluting in front and the back of the main peak in the two solvent gradients shown in Fig. 5A and B. About 55% of the material was still intact after 72 h incubation at both pH 1 and pH 10 as determined again on the analytical reverse phase C-18 HPLC. From these results we concluded that synthetic GLP-I(7–37) will be sufficiently stable during the course of the clinical studies. Indeed, we found that storage at 4° for 11 months did not give rise to any significant byproducts as determined by a chromatography on reverse phase C-18 HPLC with a gradient of triethylammonium phosphate pH 7 (buffer A) and 60% acetonitrile in buffer A (35–100%) formed in 30 min at flow rate 1 mL/min (Fig. 6). On the same column with a different gradient of 0.1% TFA/water and 0.1% TFA/acetonitrile (25–70%) formed in 30 min at 1 mL/min about 4% of byproduct was detected eluting 2 min after the desired peak.

Binding of synthetic GLP-I peptides to RIN 1046-38 cells

To extend our initial observations which showed that only two of the predicted forms of GLP-I peptides, synthetic GLP-I(7–37) and GLP-I(7–36)amide at physiological concentrations of 10^{-11} M stimulated insulin secretion in the rat perfused pancreas (23, 24), we developed binding assay using the insulin secreting RIN

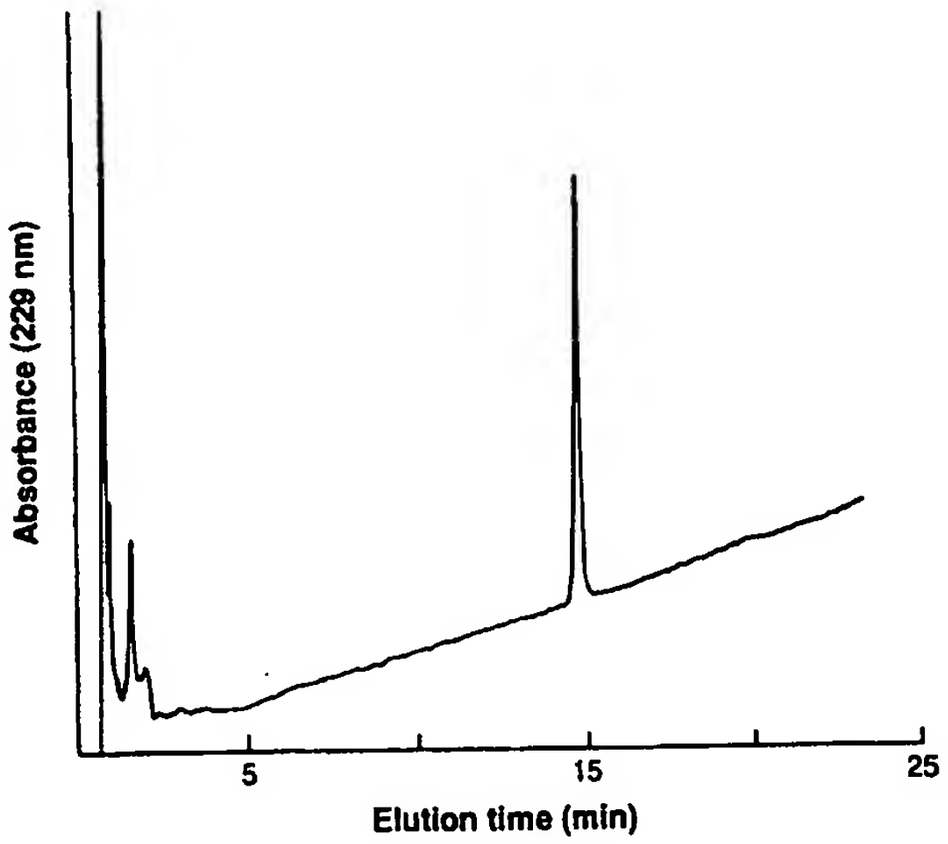


FIGURE 6

Reverse phase C-18 HPLC of pure synthetic GLP-I(7–37) (100 μg) and mannitol (500 μg) aliquoted for clinical studies. Vials were stored at 4° for 11 months. Elution was with a gradient of triethylammonium phosphate pH 7 (buffer A) and 40% of buffer A in 60% of acetonitrile (buffer B) (35–100%) formed in 30 min at flow rate 1 mL/min. In this gradient system mannitol elutes after the solvent peak in the void volume.

1046-38 cell line as a model for the pancreatic beta cell function (25). In this cell line total binding of monoiodinated ^{125}I-Tyr^{19} GLP-I(7–37) was 11% and nonspecific binding was 0.5% of the total radioactivity added, this giving specific binding of 10.5%. The relative affinity of GLP-I(7–37) for its receptor on these cells was estimated by competitive binding experiments in the presence of ^{125}I-Tyr^{19} GLP-I(7–37). Half maximum displacement of ^{125}I-Tyr^{19} GLP-I(7–37) was found at concentrations of synthetic GLP-I(7–37) of 6×10^{-10} M (Fig. 7). Similar displacement curve was obtained when synthetic GLP-I(7–36)amide was used to displace the binding. In contrast, with the longer 37-residue GLP-I(1–37) half maximum displacement was observed at much higher concentrations of 2×10^{-7} M.

To investigate the structural domains, necessary for the binding we used the carboxyl and amino terminal shortened analogs of GLP-I(7–37) in the competitive binding assay (Fig. 8). Concentrations of 3×10^{-9} M and 6×10^{-9} M of synthetic des [Gly^{37}, Arg^{36}] GLP-I(7–37) and des [Gly^{37}, Arg^{36}, Gly^{35}] GLP-I(7–37) respectively were required to obtain half maximum displacement of the bound ^{125}I-Tyr^{19} GLP-I(7–37). For the amino terminal analog des [His^{7}] GLP-I(7–37) high concentration of 2×10^{-7} M was needed to obtain the same effect.

The results from the competitive binding experiments with the three predicted forms of GLP-I and the amino and carboxyl terminal shortened analogs are in good agreement with the results obtained from the rat perfused pancreas (23, 24, 26). Together they define the structural requirements that are necessary for the expression of biological activity of GLP-I peptides in the pancreatic beta cell. First, the 37-residue long GLP-I(1–37) is the biologically inert form of the molecule which is converted to the biologically active GLP-I(7–37) and GLP-I(7–36)amide most likely through

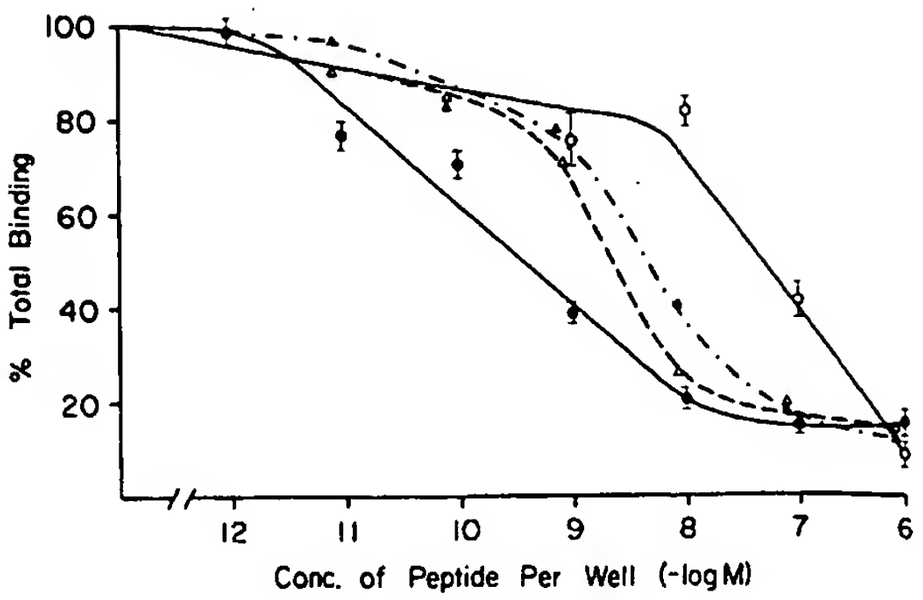


FIGURE 8

Displacement of binding of ^{125}I-Tyr^{19} GLP-I(7–37) to RIN 1046-38 cells with GLP-I(7–37) (●——●), des [Gly^{37}, Arg^{36}] GLP-I(7–37) (△– – –△), des [Gly^{37}, Arg^{36}, Gly^{35}] GLP-I(7–37) (▲–·–▲) and des [His^{7}] GLP-I(7–37) (○——○).

proteolytic cleavage at the amino terminal arginine residue at position 6. Our studies did not address the possible role of yet another form of GLP-I, the 36-residues long GLP-I(1–36)amide. However, all the experimental evidence so far supports the idea that only the shorter forms of GLP-I have a role in insulin secretion. Second, in contrast to the neuropeptides, carboxyl terminal amidation does not contribute either to the binding to or stimulation of insulin secretion from the pancreatic beta cell. Third, both the carboxyl terminal residues and the amino terminal histidine contribute to the binding and insulinotropic function of GLP-I.

The specificity of binding of GLP-I(7–37) to its receptor on the RIN 1046-38 cells was further explored by using glucagon, another insulin secretagogue in the competitive binding experiments (Fig. 9). Half maximum displacement of ^{125}I-Tyr^{19} GLP-I(7–37) binding

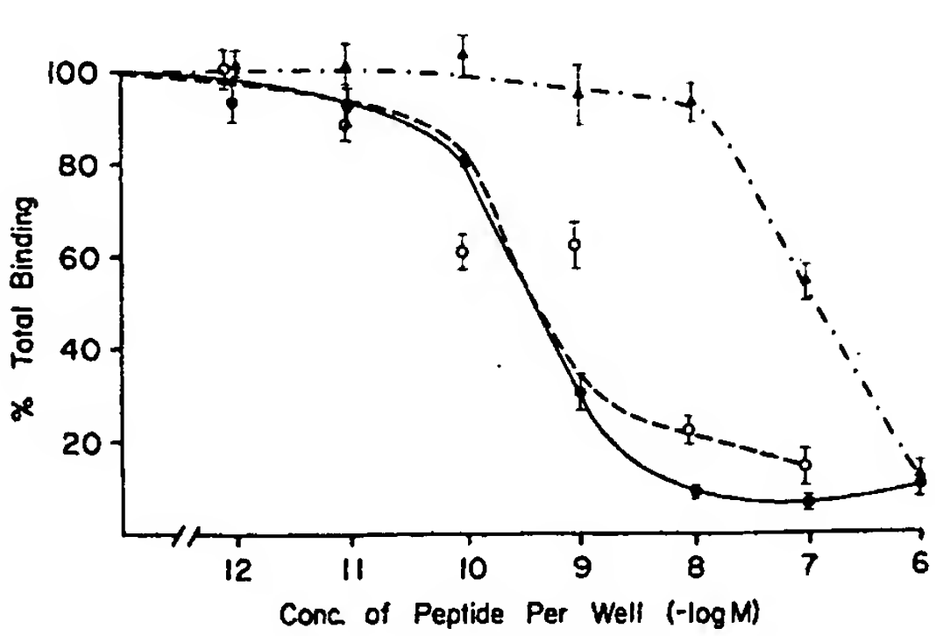


FIGURE 7

Displacement of binding of ^{125}I-Tyr^{19} GLP-I(7–37) to RIN 1046-38 cells with GLP-I(7–37) (●——●), GLP-I(7–36)amide (○– –○) and GLP-I(1–37) (▲–·–▲).

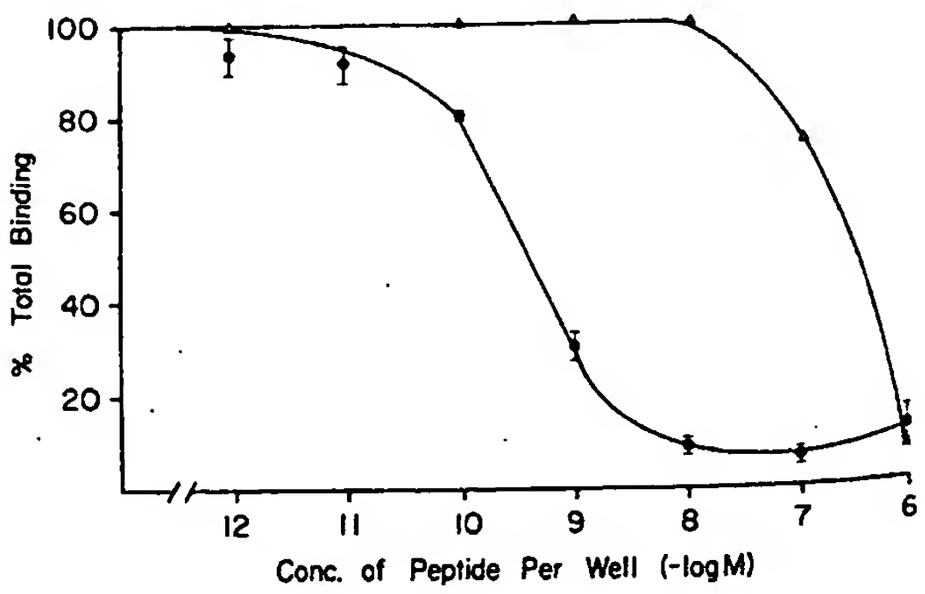


FIGURE 9

Displacement of binding of ^{125}I-Tyr^{19} GLP-I(7–37) to RIN 1046-38 cells with GLP-I(7–37) (●——●) and glucagon (△——△).

was obtained with concentrations of glucagon of 3×10^{-7} M. Thus glucagon is a weak full agonist of the GLP-I(7–37) receptor on the RIN 1046-38 cells.

The relevance of the experiments described above to human physiology was confirmed when synthetic GLP-I(7–37) was administered at physiological concentrations of 1.5 pmol/kg/min over 30 min to healthy subjects and patients with Type II diabetes mellitus. In both groups circulating levels of insulin were increased 3-fold and plasma glucose levels were decreased (27).

CONCLUSIONS

GLP-I is a new peptide. Its existence in at least three different molecular forms was deduced from the nucleotide sequence of the glucagon gene. Two of these forms of GLP-I, a 31-residue-long GLP-I(7–37) and a 30-residue-long GLP-I(7–36)amide bind to specific receptors on the pancreatic beta cell and stimulate insulin secretion and cyclic AMP formation (24, 26). GLP-I(7–37) had no effects on gluconeogenesis, cyclic AMP formation and calcium flux in rat hepatocytes and it did not antagonize the metabolic effects of glucagon as measured by these parameters (28). These data indicate that in the rat and presumably in all mammalian species the pancreatic beta cell is the main target for GLP-I(7–37) action. The findings about the specificity of GLP-I action taken together with the studies with patients with Type II diabetes mellitus (27), although preliminary and limited in its scope, raise the possibility that GLP-I(7–37) can be used therapeutically in such patients.

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